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INTRODUCTION:

Transmissible spongiform encephalopathies (TSEs) are thought to be caused by the accumulation of abnormal protease-resistant proteins called prions, which are found in aging central nervous system tissue and in the eyes. Other protease-resistant compounds, collectively called lipofuscins, also accumulate in CNS. Lipofuscins accumulate in the eye, especially in the diseased eye. An increase in lipofuscin accumulation is known to occur in human Creutzfeldt-Jakob disease victims and in other cases of experimental TSEs. Lipofuscins are fluorescent compounds with characteristic optical spectra. Some individual lipofuscin compounds (especially from the eye) have been studied in detail with regard to optical and chemical properties. The spinal cord and brain also have been observed to be fluorescent under certain wavelengths of light. This is due in part to lipofuscin accumulation in this tissue. The literature indicates that abnormal TSE prions also display characteristic optical spectra. The Principal Investigator's (PI's) preliminary data indicate that the fluorescent spectra of scrapie-infected sheep brain differ substantially from that of the noninfected sheep brain. The purpose of this study is to test the hypothesis that this spectral difference is the result of altered lipofuscin and/or prion spectral properties. Lipofuscins and prions may serve as useful fluorescent markers, which are correlated with the occurrence of TSEs and can be detected by spectroscopy.

KEY RESEARCH ACCOMPLISHMENTS:

During the first year of this study, we made only marginal progress as a result of difficulties in transmittal of funds to a collaborating laboratory. We dissected sheep and cow eyes and performed fluorescence spectroscopy on all the major eye components and reports that the cornea, lens, retina, and optic nerve show promise. Of these, the optic nerve showed the most potential for changes in spectral properties as a result of prion disease. Unfortunately, because of the lack of control tissues, the only conclusion that can be drawn is that the optic nerve shows the most intense fluorescence. The first year of this project, however, suffered from several setbacks: namely, the inability to transfer funding efficiently to the USDA collaborators and the difficulty of obtaining proper tissue samples. For example, in year one, we were forced to work under the unsatisfactory circumstances of comparing spectra from scrapie-infected sheep eyes with those from healthy cow eyes.

Year two has shown modest improvements in our working conditions. Funds were finally able to be transferred to the USDA collaborators and we were able to establish spectral comparisons between healthy and scrapie infected sheep eyes. The extent of our sampling is not, however, as large as we would like it to be and more importantly, the tissues are not age matched.

Nevertheless, our work has provided the following key results, ***as reflected in this revised progress report***:

- Spectra from the various parts of sheep eyes are very rich in detail as a function of excitation wavelength.
- Contrary to the conclusions we presented at the end of year one, it appears that while the optic nerve presents the richest spectra with the most detail, the retina is the most promising target for use as a probe. ***This conclusion is based on our recent ability to find sources of certified healthy and diseased sheep eyes. The difference between healthy and infected retinas is striking and is illustrated in Figure 1.***
- Preliminary data of total eye fluorescence from mice as a function of age are presented (Figure 2). This preliminary study anticipates the acquisition of age-matched healthy and infected tissues as well as the eventual spectroscopic sampling of various parts of the eye by confocal fluorescence microscopy.

Consequently, both tasks 1 and 2 of the Statement of Work, reprinted below continue to be addressed.

Statement of Work

Task 1.

To obtain spectroscopic data from a large statistical sampling of age-matched eyes of healthy and infected animals (mice, hamsters, sheep) in order to verify the hypothesis that TSEs may be detected by fluorescence spectroscopy. Months 1-12:

a. Obtain statistically significant samples of age-matched healthy and diseased eyes. Because lipofuscin accumulates with age, it is important to distinguish spectroscopic differences arising from age differences from those arising from TSE infection. The limiting step for this Task is the time required "to age" the subjects. All the milestones may be accomplished concurrently. Months 1-12.
b. Submit the aqueous humor, vitreous humor, lens, retina, and optic nerve to spectroscopic examination by means of steady-state fluorescence and excitation spectroscopy in order to determine whether lipofuscin fluorescence is diagnostic for TSEs. Months 1-12.
c. In so doing, determine which part of the eye, if any, is more susceptible to yielding information on TSE infection. Months 1-12.
d. Verify that no other pigments in the eye obfuscate the fluorescent signature arising from the TSEs. Months 1-12.

Task 2.

To perform an exhaustive study of the fluorescence excitation and emission spectra of solid samples and extracts from diseased and healthy eyes in order to determine the most sensitive and most reliable spectral region to exploit for probing CNS tissue. Months 1-18:

a. Characterize the fluorescence quantum yield of the lipofuscin pigment extracted from the various parts of the eye. This will require establishing a protocol that successfully removes all the fluorescent pigments from the tissue. The goal is to quantify the number of fluorescent photons that one might expect to detect per 100 incident photons and consequently begin to obtain ideas of the requisite sensitivity of the device that is the ultimate subject of Task 3. In other words, taken as a whole, this information will determine the smallest amount lipofuscin that can be monitored using a given detector and a given excitation wavelength and intensity. Months 1-12.
b. Compare the fluorescence quantum yield of the isolated pigments with that of the tissue from the eye. Months 12-18.

Task 3.

To design a prototype device to detect fluorescence from an eye *in vivo*, based upon the spectroscopic evidence accumulated. Months 18-36.

a. Depending on the results obtained from Tasks 1 and 2, we shall begin with either a green (532-nm) or blue (441-nm) laser source (both available in our laboratories). It is hoped ultimately that laser excitation will not be required because of the expense in the construction of a commercial instrument. We begin with these sources, however, in order to determine the minimum detection threshold that is required to perform a real-time investigation. It is important that the excitation intensities employed not produce damage to the eye of the subject, and these levels shall be carefully monitored. Months 18-24.
b. These results shall provide sensitivity guidelines. Detection limits will be determined, and possible signals that may interfere will be evaluated. In order to perform a real-time measurement, an optical signal should be detected in 100-300 milliseconds. Months 24-26.
c. Once these criteria have been met with the best instrumentation available to us (lasers, photomultipliers, CCDs), we shall scale down the technology to provide the most economical solution to the problem. Months 26-36.

Fluorescence spectra of healthy and scrapie-infected sheep retina are presented in Figure 1. ***The intensity axis is the same for both data sets. The inset for the healthy retinas is a blown-up vertical scale.***

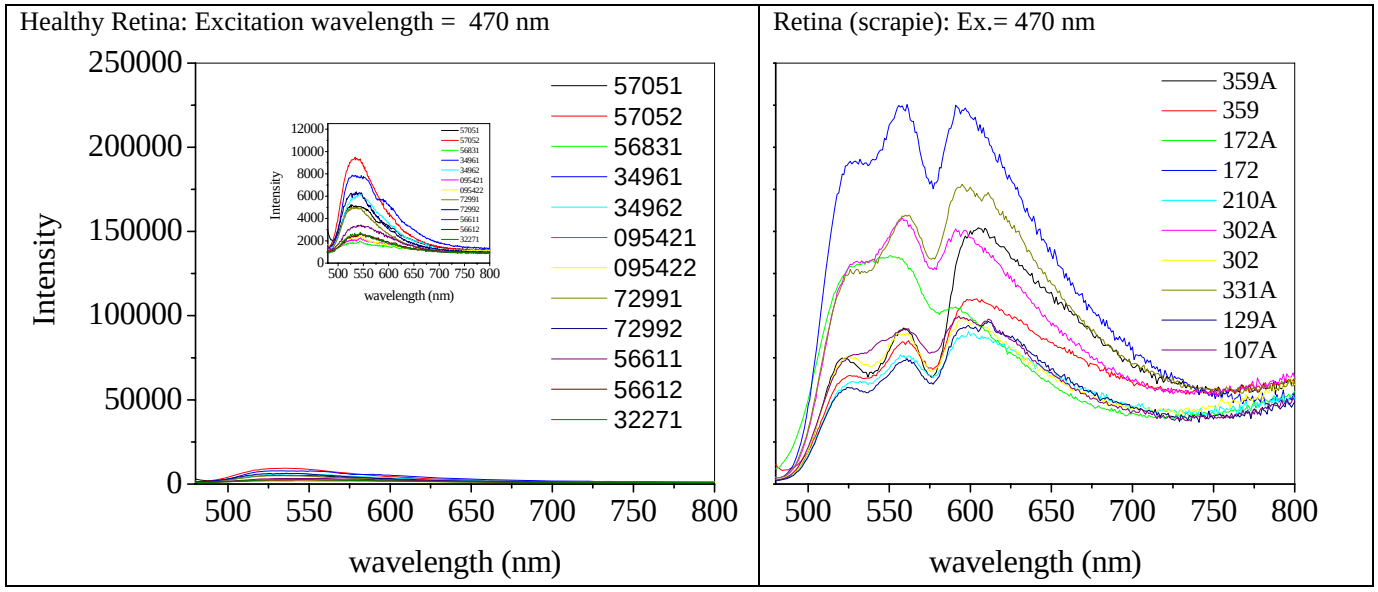

Figure 1. Comparison of healthy and infected sheep retinas at λ_{ex} = 470 nm. ***There are significant differences between the two data sets. First, the infected retinas are up to hundreds of times more fluorescent than the uninfected retinas. The structure imposed by the two peaks at ~550 and 600 nm and their relative intensities may be diagnostic. In addition, the scrapie-infected eyes present a deep trough at ~555 nm.*** In addition, we have now found a constant a reliable source of sheep eyes through our collaborators as well as through Dr. Robert G. Rowher of the VA Maryland HealthCare System, who provides scrapie infected sheep at cost.

It is evident from these data that not only are the spectral signatures of these retinas different, but that the intensity of fluorescence from the healthy retinas is much less than that of the infected retina. We are very pleased with having come to this point after all the difficulty we have had in obtaining tissues and authorizations to perform this work. What is required now is to continue to collect samples in order to have a larger statistical sampling.

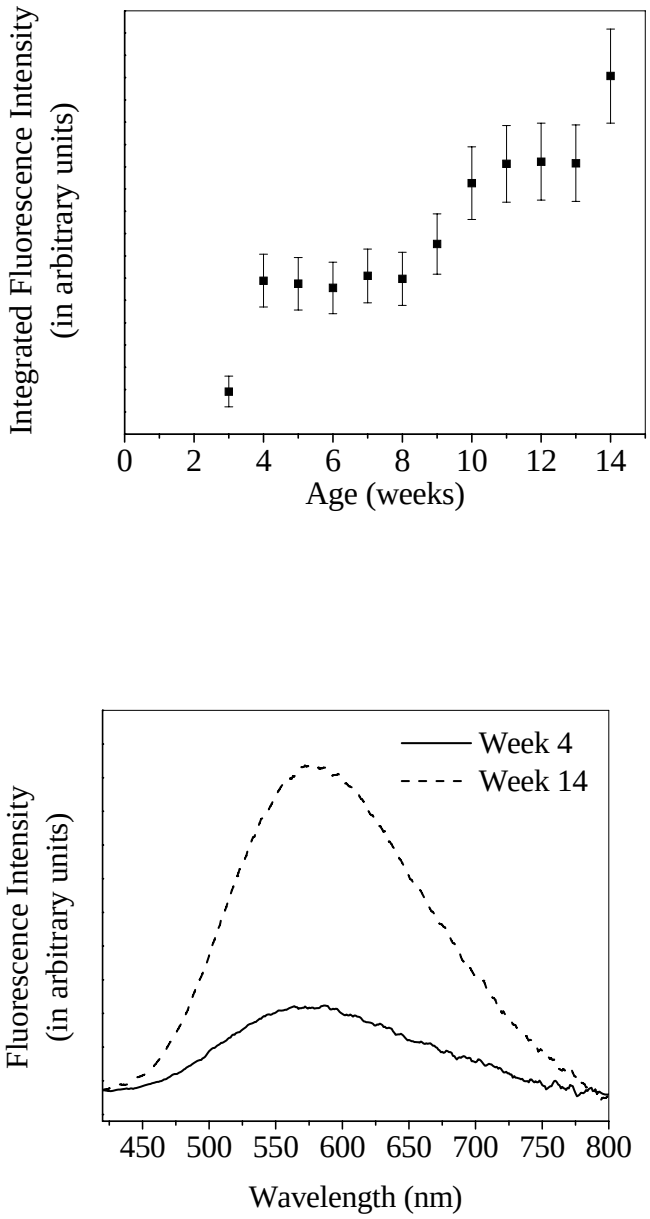

Figure 2. Total fluorescence from mice eyes as a function of age. Each eye was spread on a Fisherbrand Plain Microscope Slide of size 25 x 75 x 1-mm dimension. All samples were excited at 410 nm with a front face geometry. We did not observe any fluorescence upon excitation at 350, 470 and 520 nm respectively. An interference filter was used in the excitation side, and a long pass filter was used in the emission side to take the spectra. All data were normalized to 2 cm^2 surface area. The baseline from each spectrum was subtracted using a polynomial fit. The integrated emission intensity in the wavenumber scale were calculated after spectral smoothing. Sample data are presented for weeks 4 and 14. The error bars represent the average of 20 different experiments.

REPORTABLE OUTCOMES:

- Ms. Tessa Calhoun received her B.S. in chemistry in Spring 2005 and will be entering graduate school in chemistry at the University of California at Berkeley.
- Ms. Erin Campbell received her B.S. in biochemistry in Spring 2005 and will be taking a year off before applying to graduate schools.
- Dr. G. Krishnamoorthy is now Assistant Professor, Department of Chemistry Indian Institute of Technology, Guwahati, Assam, India.

CONCLUSIONS:

The major conclusions of the work executed so far are that specific parts of sheep eyes have been identified that may provide spectroscopic signatures of prion disease: these are the retina, lens, and sclera. Surprisingly, the optic nerve did not provide spectroscopic differences between healthy and infected tissue, as was anticipated in the year 1 report. All parts of the eye have been investigated. We note, however, that the samples are not age matched. Given the reproducibility of the spectral features for retina, lens, and sclera, this may prove to be a positive aspect since the results of Figure 2 demonstrate the increase of autofluorescence from eyes as a function of age. Specific wavelengths have been identified for exciting and detecting useful fluorescence signatures.

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APPENDICES:

None.